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(54) Title: CONDITIONAL TRANSFORMATION OF GENETICALLY ENGINEERED CELLS

(57) Abstract

This invention concerns materials, methods and applications relating to the multimerizing of chimeric proteins with a dimeric or multimeric, preferably non-peptidic, ligand. Aspects of the invention are exemplified by ligand-mediated transformation of stem cells permitting stem cell growth in a growth-factor independent manner.

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Conditional Transformation of Genetically Engineered Cells

Technical Field

This invention concerns materials, methods and applications relating to the multimerizing of chimeric proteins with a dimeric or multimeric, preferably non-peptidic, ligand. Aspects of the invention are exemplified by ligand-mediated transformation of cells permitting cell growth in a growth-factor independent manner.

Background

The difficulty of obtaining bone marrow that is immunologically compatible with the host has greatly limited the usefulness of bone marrow transplantation. Improperly matched bone marrow leads to complications that are frequently costly and debilitating and in about 20% of cases lethal. Most of these complications could be avoided with the use of autologous bone marrow. For this reason there has been great focus on the development of methods to grow human bone marrow in culture. Such efforts have had only limited success because of the unusual way that bone marrow stem cells proliferate. To date, stem cell growth factors that are able to maintain a truly pluropotential stem cell in culture have not been found. So called stem cell growth factor actually promotes the proliferation of cells that are several steps more differentiated than a true stem cell and hence are committed to a particular hemopoetic lineage.

Meanwhile, independent of clinical research in cancer therapies, biological switches have been developed which are based on chemically induced dimerization of proteins. Aspects of that work are disclosed in Spencer et al, 12 Nov 1993, Science 262:1019-1024 and International Patent Applications PCT/US93/01617 and PCT/US94/08008, the contents of all three of which are incorporated herein by reference.

Described herein are methods and materials which can be used, *inter alia*, to maintain, *via* such chemically induced dimerization of proteins, the proliferation of cells, such as stem cells, in culture for the adjuvant treatment of malignancy. These approaches can be used to induce the conditional growth of a variety of cell types and to render the progeny of hematopoietic stem cells, T cells and macrophages in particular, resistant to HIV by targeting essential viral proteins to the cellular degradative pathway.

Illustrative publications disclosing further background information of interest are provided in PCT/US93/01617, especially on pages 1-4. However, as will be clear from this disclosure, none of the foregoing authors describe or suggest the present invention.

Summary of the Invention

This invention provides materials and methods for the genetic engineering of host cells to render the cells and their progeny susceptible to conditional transformation. Preferably the cells are of mammalian origin, more preferably of human origin, and are not terminally differentiated, e.g. stem cells such as hematopoietic stem cells or skin cells. Such genetic engineering and the process of conditional transformation are useful, e.g., for expanding a population of such cells. The engineered cells, as well as organisms containing them, are useful in clinical applications and as biological reagents for a variety of research and production purposes.

The invention involves the adaptation of methods and materials for using homo- and hetero-multimerization of chimeric "responder" proteins to trigger gene transcription or other biological events in living cells. (As used herein, the terms multimer, multimerize and multimerization encompass dimers, trimers and higher order multimers and their formation.) The chimeric responder proteins are intracellularly expressed fusion proteins which contain one or more specific receptor domains, e.g., FK506 binding protein (FKBP) domains, capable of binding to a corresponding multimerizing agent (e.g. an FK1012 molecule in the case of FKBP domains). The multimerizing agent is a multivalent ligand which is capable of binding to receptor domains on more than one of the chimeric protein molecules. Such binding to more than one of the chimeric protein molecules yields dimers or higher order multimers of the chimeras. These chimeric proteins are thus multimerizable by virtue of containing a binding domain for a corresponding multivalent ligand. The multimerizable chimeras contain one or more additional domains, in addition to the receptor domain(s). The additional domain(s) may comprise a DNA binding domain, a transcriptional activating domain, a membrane targeting domain (e.g. a myristoylation signal), a cellular destruction domain, a domain such as a single chain antibody (ScFV) or other domain. The chimeric proteins are designed such that ligand-mediated multimerization triggers a biological event such as transcription of a transforming gene under the transcriptional control of a DNA element responsive to such multimerization, destruction of a tumor suppressor or viral protein, or direction of a transforming gene product to the cellular site where it assumes transforming activity such as the nucleus or cell membrane.

Ligands and ligand binding domains as well as other components and methods for regulated transcription and other biological events which may be adapted for use in various

embodiments of this invention are disclosed in detail in International Patent Applications PCT/US93/01617 and PCT/US94/08008, as well as in Spencer et al, Science, 1993, the contents of which are incorporated herein by reference.

Brief Description of the Figures

Figure 1. (A) Construction of chimeric soluble and membrane-bound Sos molecules. The plasmid coding sequence for the full length hSos, containing amino acid residues 2 to 1333 was cloned immediately downstream of the v-Src myristoylation targeting domain (residues 1-14) in the Xho 1-Sal 1 site of a derivative of the eukaryotic expression vector pBJ5 (Spencer et al. (1993) Science 262, 1019-1024; Pruschy et al (1994) Chemistry & Biology 1, 163-172) to yield MSosE. The soluble construct SSosE was constructed by placing hSos in an identical pBJ5-derived vector lacking the myristoylation sequence. The FKBP12 module derived from hFKBP (Gulbins et al (1993) Science 260, 822-825) was amplified by PCR as described (Spencer et al.,1993, *supra*) and cloned in three tandem copies into the Xho 1 site of SSosE to yield SF3SosE. This FKBP12 module was cloned in three tandem copies downstream of the myristoylation targeting domain in the vector described above to yield MF3E. All constructs contained a C-terminal influenza HA epitope tag to facilitate protein detection. Integrity of the constructs were verified by dideoxy sequencing. S, Sal 1; X, Xho 1; Sc, Sac II; E, Eco R1. **(B) Activation of T cell signal transduction by membrane-targeted Sos.** Jurkat-TAg cells (10^7) were transfected with 2 μg NF-AT-SX secreted alkaline phosphatase (SEAP) reporter plasmid and either 8 μg of MSosE, SSosE, 4 μg of constitutively active v-Ha-Ras or dominant negative Ras RSVN17Ras), or vector alone control. In all transfections, equal molar amounts of each plasmid were transfected and vector DNA used to keep the amount of DNA transfected constant. 24 hrs post transfection, aliquots of 10^5 cells from each transfection were stimulated for an additional 20 hrs with media alone, ionomycin (1 μM), or ionomycin plus PMA (25 ng/ml) in 200 μl of growth media. Following stimulation, media was examined for SEAP activity (Gulbins et al (1994) Mol. Cell Biol 14, 906-913). The data are presented as percent activation following stimulation with ionomycin alone relative to cells from each transfection stimulated with ionomycin + PMA (100%). The data represent the average of at least three independent experiments and are plotted as mean + SEM. Inset: To verify expression of the constructs, samples from the above transfections were lysed in RIPA buffer, immunoprecipitated using the 12CA5 mAb specific for the HA epitope tag and analyzed by SDS-PAGE followed by immunoblotting. **(C) Myristoylated Sos synergizes with calcium-dependent signaling events.** Jurkat-TAg cells were cotransfected with 2 μg NFAT-SX, 0.2 μg constitutively

active calcineurin CNMUT2B (Clipstone, N.A. & Crabtree, G.R. (1993) Annals New York Acad. Sci. 696, 20-30; O'Keefe et al (1992) Nature 357, 692-695.), and either 4 μg v-Ha-Ras, 8 μg MSosE, SSosE, or vector alone. Stimulation and analysis for SEAP activity was done as described in (B). Activation is presented as percent following stimulation with media alone relative to activation following stimulation with PMA on samples for each transfection. **(D) Membrane-targeted Dbl and Vav do not activate signal transduction in T cells.** Jurkat-TAg cells were cotransfected with 2 μg of NFAT-SX reporter plasmid and 8 μg the indicated expression plasmids MDblE, McVavE, and ScVavE, stimulated, and analyzed for SEAP activity as described in (B). MDblE contains amino acid residues 2 to 498 from oncogenic Dbl and McVavE and ScVavE contain residues 2 to 845 of the full length Vav proto-oncogene, cloned into the myristoylated and soluble vectors as described in Figure 1A. Activation is again presented as percent following ionomycin stimulation alone relative to cells stimulated with ionomycin + PMA. Inset: Expression of epitope-tagged constructs verified as in (B). Asterisk denotes a nonspecific band recognized by the 12CA5 antibody.

Figure 2. Soluble and myristoylated Sos exhibit distinct subcellular localization. Cos-1 cells were transfected on coverslips with SSosE (A,B) or MSosE (C,D) expression plasmids, fixed and stained with 12CA5 mAb and FITC-conjugated rabbit anti-mouse secondary antibody, and analyzed by confocal microscopy. MSosE localizes predominantly to the plasma membrane while SSosE remains cytosolic.

Figure 3. (A) Construction of Sos molecules containing mutations in the C-terminal proline-rich regions. Using PCR-mediated site-specific mutagenesis, one or both of the proline-rich sequences containing amino acids shown were changed to alanines as illustrated to abolish the consensus for SH3 binding. The location of these mutations in reference to the catalytic domain and pleckstrin homology domain (PH) is shown. The altered versions of Sos were cloned into the vector described in Figure 1 to generate MSosP1E and MSosP1,2E. **(B) Membrane-bound Sos molecules with mutant C-terminal proline-rich domains no longer bind to Grb-2.** Jurkat-TAg cells were cotransfected with the illustrated membrane-bound Sos constructs or vector alone with excess soluble Grb-2 cloned into the pBJ5 vector for high expression levels, immunoprecipitated in duplicate with 12CA5 antibody, and analyzed by SDS-PAGE. Samples were immunoblotted with either 12CA5 mAb to detect Sos constructs or anti-Grb-2 mAb to detect the presence of coprecipitated Grb-2. **(C) Activity of membrane-bound Sos is independent of interaction with Grb-2.** Jurkat-TAg cells were cotransfected with 2 μg NFAT-SX, and 8 μg of the illustrated

MSosE mutants. Stimulation and analysis for SEAP activity was done as described in Figure 1. The data are presented as percent activation following stimulation with ionomycin relative to activation following stimulation with ionomycin + PMA for each transfection.

Figure 4. (A) Using FK1012 to mimic the role of Grb-2-induced localization of Sos. Upper panel, the proposed physiologic role of Grb-2 to localize Sos to the cell membrane. Lower panel, FK1012 induces the localization of a chimeric Sos/FKBP to a myristoylated FKBP at the cell membrane. (B) Activation of Ras by inducible membrane localization of Sos. Jurkat-TAg cells cotransfected with 2 μg NFAT-SX, 9 μg SF3SosE or 4.5 μg (molar equivalent) SF3E, and 1 μg MF3E. Twenty-four hours following transfection, cells were stimulated with ionomycin plus the illustrated dilutions of FK1012 for 20 hrs and cell supernatants analyzed for SEAP activity as described in Figure 1. The data are presented as percent activation following stimulation with ionomycin + FK1012 relative to activation following stimulation with ionomycin + PMA on samples for each transfection. Expression of all FKBP-containing constructs was confirmed by immunoblotting. (C) Activation of Ras by inducible membrane localization of Sos is inhibited by dominant negative Ras. Ionomycin plus the illustrated dilutions of FK1012 were added to Jurkat-TAg cells cotransfected as described in (B) with either 4 μg dominant negative Ras or 4 μg vector control. Activation is presented as described in (B). (D) Inducible localization of Grb-2 does not activate Ras. Ionomycin plus the illustrated dilutions of FK1012 were added to Jurkat-TAg cells cotransfected with 2 μg NFAT-SX, 4 μg SF3GrbE, or 4 μg SF3E, and 1 μg MF3E. Activation is presented as described in (B).

Fig 5. (A) Construction of chimeric intracellular signaling molecules. Schematic of Src-family kinase-FKBP12 chimeras used for inducible membrane targeting. Src-family kinases are deregulated by mutation of the C-terminal tyrosine residue as shown and inactivated by truncation of the N-terminal myristoylation targeting peptide (residues 1-10). SF1ΔSH3Fyn lacks residues 1 - 144, SF1ΔSH3,SH2Fyn lacks residues 1 - 254 and SF1ΔKFyn has substitution K296E. Membrane targeting is achieved by subcloning these modified kinases into the FKBP12-tagging vector MF1E (myristoylated) or SF1E (cytosolic). M, myristoylation targeting sequence from v-src (residues 1-14) (Spencer et al.,1993, *supra*; Cross, 1984, MCB); S, soluble, nonmyristoylated; U, unique domain; E, influenza hemagglutinin epitope tag (Field et al, 1988, MCB); TAIL, C-terminal regulatory peptide. (B) Model of the regulation of Lck/Fyn by the CD45 protein-tyrosine phosphatase and Csk PTK. Removal of the C-terminal phosphate by the CD45 protein tyrosine phosphatase relieves Lck/Fyn tyrosine kinase inhibition, permitting

downstream signaling (modified from Weiss, A. Y Littman, D. R. (1994) Cell 76, 263-274). (C) Model of inducible membrane targeting with synthetic ligands. (Left) SRC-family kinases have been inactivated by the replacement of their membrane targeting motifs by FKBP12. (Right) With the addition of membrane-permeable FK1012, the tyrosine kinases are recruited to the plasma membrane docking protein MF1, eliciting a biological response. Y, tyrosine; YP, phosphotyrosine.

Fig 6. Inducible signal transduction using synthetic dimers by membrane targeting of Src-family kinases. (A) The ability of dimeric ligand FK1012 to recruit SF1Fyn to the plasma membrane docking protein MF1E is assayed by the induction of an NF-AT-responsive reporter plasmid NF-AT-SX in Jurkat-TAg cells (Clipstone and Crabtree, Nature, 1992; Northrup et al, 1993, Nature). This is compared to the FK1012-dependent recruitment of wild-type Fyn (SF1Fynwt) or cytosolic FKBP12 (SF1E). (B) The ability of FK1012 to target multiple Src family members to the plasma membrane docking protein MF3E compared to control protein SF1E. (C and D). The signaling capacity of various Fyn (C) or Lck (D) mutants lacking their SH3 (ΔSH3), SH3 and SH2 (ΔSH3,2) or kinase (ΔK) domain is compared to the parent constructs SF1Fyn (C) or SF1Lck (D) by membrane targeting with FK1012. As a control, CID-independent signaling by myristoylated FKBP-Lck (MF1Lck) is shown. All of the constructs were similarly expressed as assayed by Western blot using the 12CA5 mAb against the influenza hemagglutinin epitope (Cross, 1984, MCB).

Fig 7. Conditional activation of Src-family kinases mimics TCR signaling. Comparison of the induction of a panel of transcription factors (Spencer et al.,1993) by CID-induced Fyn (A) or Lck (B). (C) The ability of dominant-negative Ras (RSV-N17Ras, 2 μg) or FK506 (2 ng/ml) to block NF-AT activation by membrane recruitment of Fyn or Lck by 300 nM FK1012. (D) An examination of the kinetics of activation by Fyn and Lck using 1 μM FK1012 relative to that by mitogen or direct TCR crosslinking (see Materials and Methods).

Fig 8. Membrane recruitment of SF1Fyn activates signaling independent of the TCR complex. (A) The signaling capacity of Fyn was examined in the TCR- variant of Jurkat cells, J.RT-T3.5 (Weiss, A. & Stobo, J. (1984) J. Exp. Med, 160, 1284-1299), by assaying the induction of NF-AT. (B) The signaling capacity of Fyn or kinase-deficient Fyn (SF1FynΔK) was examined in non-T COS cells by assaying the induction of AP-1. (Inset) Western blot of samples from the above transfections using 12CA5 anti-hemagglutinin mAb.

Regulated Expression Of One Or More Transforming Genes

In certain embodiments of this invention, the cells are engineered to contain and be capable of expressing one or more transforming genes under the expression control of a transcriptional control element responsive to the presence of a predetermined ligand, e.g. by adaptation of the regulated transcription technology disclosed in International Patent Applications PCT/US93/01617 and PCT/US94/08008 and in Spencer et al, Science, 1993.

Transforming genes, as that phrase is used herein, may be of two types. The first are DNA sequences encoding transforming proteins such as myc, fos, myb, etc. whose transforming activity is attributed to their overexpression. These transforming genes may be linked to a desired transcriptional regulatory element for regulatable expression as discussed below. The second type of transforming genes encode proteins such as ras, raf, sos or src-like tyrosine kinases, the transforming activity of which is attributed to their localization to the cell membrane and/or allosteric changes that can be induced by physical proximity of an activated protein. In the case of Src-like kinases, the DNA sequence comprising the transforming gene encodes a protein containing at least the activated form of the kinase portion of the src-family member. Transforming genes of the second type, e.g. ras, raf, sos, activated kinase, etc., are regulatably expressed as chimeric genes linked to a DNA sequence encoding a cellular targeting domain. The transforming gene product in such cases comprises a fusion protein containing the peptide sequence encoded by the transforming gene fused to a targeting domain such as a nuclear localization sequence or a myristoylation sequence which targets the fusion protein to the cell membrane.

Regulatable expression involves recombinant DNA constructs ("target gene constructs") containing a first DNA sequence encoding a transforming gene (e.g. myc or a fusion protein of sos linked to a myristoylation sequence), and a second DNA sequence comprising a transcriptional regulatory element, such as a promoter or enhancer sequence, which is responsive to the multimerization of chimeric responder proteins. Target genes of these embodiments comprise transforming genes, as discussed below.

DNA sequences for the desired transforming genes may be readily obtained by conventional means. For instance, primers may be designed based on the published sequence of a desired target cDNA, synthesized by conventional procedures and used in obtaining target gene DNA through standard PCR techniques. DNA sequence information and other information relevant to the cloning and use of transforming gene sequences are readily available.

This aspect of the invention involves the use of one or more chimeric responder proteins, DNA constructs ("responder" constructs) encoding them, and multi-valent ligand molecules

capable of multimerizing the chimeric proteins. These are described in detail in the cited patent documents above. Briefly, the chimeric proteins contain at least one ligand-binding (or "receptor") domain and an action domain capable, upon multimerization of the chimeric responder molecules, of initiating transcription of the transforming gene within a cell. The chimeric proteins may further contain additional domains. These chimeric responder proteins and the responder constructs which encode them are recombinant in the sense that their various components are derived from different sources, and as such, are not found together in nature (i.e., are mutually heterologous). Also provided are recombinant transforming constructs containing a transforming gene under the transcriptional regulation of a transcriptional control element responsive to the presence of the multimerizing agent, i.e, to multimerized responder proteins described above. The transcriptional control element is responsive in the sense that transcription of the transforming gene is activated by the presence of the multimerized responder chimeras in cells containing these constructs. Said differently, exposure of the cells expressing the chimeric responder constructs and containing a transforming gene construct responsive to the multimerizing ligand results in expression of the transforming gene. The constructs of this invention may contain one or more selectable markers such as a neomycin resistance gene (neor) and herpes simples virus-thymidine kinase (HSV-tk). When genetically engineered cells of this invention which contain and express the responder constructs, and contain the transforming gene construct, are exposed to the multimerizing ligand, expression of the transforming gene is activated.

To produce the modified cells one introduces the desired construct(s) into selected host cells. This may be accomplished using conventional vectors (various examples of which are commercially available) and techniques. If desired, the modified cells into which one or more constructs have been successfully introduced may then be selected, separated from other cells and cultured, again by conventional methods.

The multimerizing ligands useful for triggering the expression of the transforming gene in the practice of this invention are capable of binding to two (or more) of the receptor domains, i.e. to two or more chimeric responder proteins containing such receptor domains. The multimerizing ligand may bind to the chimeras in either order or simultaneously, preferably with a Kd value below about 10-6, more preferably below about 10-7, even more preferably below about 10-8, and in some embodiments below about 10-9 M. The ligand preferably is a non-protein and has a molecular weight of less than about 5 kDa. Even more preferably, the multimerizing ligand has a molecular weight of less than about 2 kDa, and even more preferably, less than 1500 Da.

The design and use of chimeric responder proteins capable, upon ligand-mediated multimerization, of triggering transcription of a target gene, as well as the design, construction

and use of DNAs encoding them, are disclosed in PCT/US94/01617 and in Spencer et al, Science, 1993.

Briefly, the action domains of the chimeric proteins may be selected from any of the proteins or protein domains (preferably of the species of the desired host cells or organism) which upon multimerization are capable of activating transcription of a target gene which is under the transcriptional control of a cognate control element. For instance, the action domain of the chimeric responder protein molecules may comprise a protein domain such as a CD3 zeta subunit which is capable, upon exposure to the ligand and subsequent multimerization, of initiating a detectable intracellular signal leading to transcriptional activation via the IL-2 promoter. Alternatively there may be a series of responder proteins, e.g. in which one contains as its action domain, a DNA-binding protein such as GAL4 while another contains as its action domain a transcriptional activation domain such as VP16. Heterodimerization of such responder proteins to form a GAL4-VP16 dimer activates the transcription of genes (in our case, the transforming gene) under the transcriptional control of elements to which the (hetero)dimerized responder proteins can bind. In all such embodiments, multimerization activates transcription of the transforming gene under the transcriptional control of a transcriptional control element (e.g. enhancer and/or promoter elements and the like) which is responsive to the multimerization event.

DNA constructs for the various embodiments of this invention may be assembled in accordance with the design principles, and using materials and methods, disclosed in the patent documents cited herein, including PCT/US94/01617, with modifications as described herein and as disclosed in the examples which follow

This invention further involves DNA vectors containing the various constructs described herein (for these and other embodiments), whether for introduction into host cells in tissue culture, for introduction into embryos or for administration to whole organisms for the introduction of the constructs into cells in vivo. In either case the construct may be introduced episomally or for chromosomal integration. The vector may be a viral vector, including for example an adeno-, adeno associated- or retroviral vector. The constructs or vectors containing them may also contain selectable markers permitting selection of transfectants containing the construct.

This invention further encompasses the genetically engineered cells containing and/or expressing the constructs described herein, including prokaryotic and eucaryotic cells and in particular, yeast, worm, insect, mouse or other rodent, and other mammalian cells, including

human cells, of various types and lineages, whether frozen or in active growth, whether in culture or in a whole organism containing them.

The genetically engineered cells of such embodiments may contain and be capable of regulatably expressing more than one such transforming gene (e.g. myc and a sos-myristoylation sequence fusion), each of which may be under the same or different multimerizer-regulated expression control. Exposure of the engineered cells or their progeny to the multimerizing ligand(s) recognized by the chimeric transcription control proteins results in expression of the transforming gene(s) and in cellular growth characteristic of a transformed phenotype.

Regulated Localization Of A Transforming Gene Product

In other embodiments the transforming genes may be used in a conditionally transforming manner, i.e. where the peptide sequence encoded by a transforming gene of the second type e.g., ras, raf, sos, activated kinase, etc., is fused to a ligand-binding domain, such as an FKBP domain. The ligand-binding domain confers targeting capabilities to the transforming gene product. The resultant fusion proteins are capable of ligand-mediated association with a membrane docking protein or other localization protein, and thus constitute a targetable transforming factor. Localization proteins are fusion proteins containing a ligand-binding domain and a targeting domain which directs the fusion protein to a particular cellular location, e.g. the cell membrane in the case of a myristoylation sequence or the nucleus in the case of a nuclear localization sequence, for example. In the presence of an apppropriately chosen multivalent ligand capable of binding to two or more ligand-binding domains, the targetable transforming factor is directed to the desired cellular location by association with the localization protein.

In such embodiments, the cells are engineered to contain and be capable of expressing recombinant DNA sequences encoding one or more targetable transforming factors and localization protein(s). The first DNA sequence encodes a chimeric protein comprising a ligand binding domain fused to a peptide sequence encoded by a transforming gene, which can, upon localization to the appropriate cellular environment (e.g. the nucleus or, in the case of proteins such as raf or sos, the cell membrane), activate a transformation pathway. The second DNA sequence encodes a chimeric protein comprising a cellular or subcellular localization domain (e.g. a myristoylation site targeting the protein to the cell membrane, or a nuclear or other localization signal) fused to a ligand-binding domain. In the presence of a multimerizing ligand, the chimeric transforming factor molecules multimerize with the chimeric localization proteins and thus become localized at the cell membrane or other targeted site. The DNA sequences encoding the two chimeric proteins may themselves be expressed in a ligand-regulated manner as described above, using the

same or different ligand to which the chimeric transforming and localization proteins bind. The cells may further contain at least one transforming gene encoding a protein such as myc, fos, myb, etc. whose transforming activity is attributed to an overexchange mechanism, which transforming gene may be expressed constitutively or under the control of the ligand-regulated system alluded to above, under the regulation of the same or different ligand to which the chimeric proteins above bind.

To illustrate this aspect of the invention, we have prepared an activation construct encoding a human SOS protein fused to multiple FKBP domains. A second construct encoding a membrane docking protein was prepared which encodes the 20 amino acid myrsitoylation signal from the c-src protein fused to multiple FKBP domains. When these two constructs are transfected into Jurkat T lymphocytes by electroporation and the dimerizing agent FK1012 added to the engineered cells, there is rapid and complete activation of the transformation mechanisms with the cells as demonstrated by the activation of AP-1 and NF-AT directed transcription.

In another illustration, constructs were prepared which encode a targetable transforming factor comprising a fusion protein containing a number of FKBP domains and a modified src-family tyrosine kinase such as fyn, lck, lyn, etc. The modifications to the src-family tyrosine kinase included incorporation of known transforming mutations, deletion of the myristoylation sequence which is required for transforming activity, and optional deletion of other domains including all non-kinase domains. Host cells were then transfected with such a construct and with a construct encoding an FKBP-containing membrane docking protein. In the presence of the dimeric synthetic ligand, FK1012, the targetable transforming factors associate with the membrane docking protein and are thus directed to the cell membrane where they activate the transforming process. This is evidenced by the development of transcriptional activation of transforming proteins such as AP-1 and others.

Regulated Destruction Of Tumor Suppressors

In other embodiments of this invention, the cells are engineered to contain and be capable of expressing recombinant DNA sequences encoding chimeric proteins comprising various combinations of one or more of the following domains: a target binding domain (such as a single chain FV or other antibody moiety), a multimerizing ligand-binding domain, and a domain targeting the chimera for degradation or destruction.

One such embodiment involves cells engineered to contain two such rDNA sequences. The first DNA sequence encodes a first chimeric protein containing a ligand-binding domain and a tumor suppressor binding domain. The second such DNA sequence encodes a second chimeric

protein containing a ligand-binding domain (which may be the same or different from the ligand-binding domain of the first chimera) and a domain targeting the chimera for degradation or destruction. In the presence of a multimerizing ligand capable of binding to both the first and second chimeras, the chimeras multimerize. The tumor suppressor to which the first chimera binds is thus linked in trans to the degradation targeting domain and is thereby targeted for destruction and effectively removed from the engineered cells.

Tumor suppressors to be targeted in such embodiments of this invention include p15, p16, p21, p27, Rb and the like. See e.g. Weinberg, 1991, "Tumor Suppressor Genes" Science 254:1138-1146. Degradation targeting domains include domains such as the cyclin destruction box and the jun degradation signal. Tumor suppressor binding domains may be readily prepared in the form of single chain FV fragments (ScFV's) capable of recognizing the relevant tumor suppressor.

In other embodiments, the cells are engineered to contain a DNA sequence encoding a chimeric protein containing a target binding domain such as a ScFV directed to the desired target and a domain targeting the chimera for degradation or destruction, e.g. via a proteolytic pathway. That DNA sequence is linked to and under the expression control of a transcriptional control element responsive to the presence of a predetermined ligand, e.g. by adaptation of the regulated transcription technology disclosed in International Patent Applications PCT/US93/01617 and PCT/US94/08008, as as discussed above.

Regulated Degradation Of Viral Components

The two approaches to regulated destruction described above may be extended from targeting one or more tumor suppressors to targeting one or more viral proteins, and in particular, one or more essential proteins of an HIV virus, for example. In such cases, recombinant DNA sequences encoding chimeras containings ScFV's are used as above, but directed to HIV proteins such as the HIV protease, nef of others. Introduction of the recombinant DNA molecules into hematopoietic stem cells provides a route to macrophages and T cells capable of expressing the recombinant DNAs. Such cells contain the ligand-regulated system for degradation of the targeted viral proteins, and in that sense, would be characterized by ligand-induced resistance to the virus.

Ligands and Components Of DNA Constructs

Various DNA sequences for incorporation into recombinant DNAs of this invention may be obtained as described in PCT/US94/1617. Those include DNA sequences encoding cellular localization signals (such as myristoylization sites for directing chimeras to the cell membrane)

and DNA encoding ligand binding domains (including naturally occurring or genetically engineered FKBPs or cyclophilins).

ScFV's may be produced by conventional methods using cloned DNA encoding portions of antibodies against the desired tumor suppressor (or viral protein or other target), which may also be prepared by conventional methods. For instance, using conventional methods one may obtain mAbs which specifically recognize a desired tumor suppressor. Starting with murine hybridoma or spleen cells which produce such antibodies, one may generate phage which contain DNA encoding the desired ScFV using the commercially available Recombinant Phage Antibody System and pCANTAB 5 Gene Rescue and Sequencing Primers (Pharmacie Biotech)(or the equivalent). See Analects 22(1):1-7 (Winter 1993) and references cited therein. DNA encoding the desired ScFV may be readily linked at its 3' end to DNA encoding the ligand-binding domain to form a recombinant DNA encoding the chimeric protein mentioned above which comprises a ligand binding domain and a tumor suppressor binding domain.

Degradation targeting domains such as the jun and cyclin destruction boxes or a ubiquitin conjugating enzymatic domain, for example, may be cloned via PCR or synthesized using automated oligonucleotide synthesis procedures.

A wide variety of transforming genes are known which may be obtained from the ATCC, by cloning (PCR) or by assembly of overlapping synthetic oligonucleotides. See e.g. McCormick, "ras Oncogenes" pp125-145; Hunter, "Oncogene Products in the Cytoplasm: The Protein Kinases", pp147-173; Eisenman, "Nuclear Oncogenes" pp 175-221, and other chapters in Oncogenes and the Molecular Origins of Cancer (Cold Spring Harbor Press, 1989, Weinberg, ed.).

Design And Assembly Of DNA Constructs

DNA sequences encoding the various components may be assembled into recombinant DNA molecules encoding the desired chimeras by analogy to the methodology described in PCT/US94/1617. The recombinant molecules may be assembled or transferred into vectors for propagation or transfection which may additionally contain transcriptional control elements such as the desired promoter/enhancer elements and conventional genetic elements such as origins of replication and selection markers.

Cells

Any eukaryotic cells may be engineered in accordance with this invention. However, hematopoietic stem cells of mammalian origin, e.g. murine or preferably primate, and in

particular human origin, are preferred. Cells are obtained, manipulated and cultured using methods conventional for the respective cell type and origin.

Introduction Of Constructs Into Cells

Conventional methods such as electroporation, liposome transfer, DEAE transfection and calcium phosphate transfection may be used for introducing the constructs into the cells. Transfectants are selected using conventional methods and materials. The cells are then cultured in a medium containing an amount of the multimerizing ligand effective for conditional transformation or for cell growth. Cells may then be washed or otherwise manipulated and ultimately transferred, in the case of stem cells, using established techniques for bone marrow transplantation.

Uses: Clinical And Non-Clinical

Populations of conditionally transformed autologous (CTA) stem cells of this invention may be expanded by culture in a culture medium containing the multimerizing ligand in an effective amount for growth of the cells. Cells so produced may be administered to a patient in need thereof as an adjuvant to cancer chemotherapy, e.g. for leukemia, lymphoma and various solid tumors as an alternative to bone marrow transplantation with donated bone marrow cells. Bone marrow transplantation may also be effected using CTA stem cells rendered resistant to HIV as described above. Administration to the patient of the multimerizing ligand in an amount effective to cause multimerization of the chimeric proteins present in the engineered cells renders those cells and their progeny resistant to HIV. Also, epithelial cells may be engineered to impart conditional transformation characteristics in accordance with this invention. Culture of such cells is then effected in media containing the multimerizing ligand in an amount sufficient to permit cell growth. Cells so produced may then be transplanted with CTA cutaneous stem cells.

The methods and materials of this invention may also be used for non-clinical purposes. For instance, they may be used to produce expanded populations of stem cells for providing to the research community for the study of asymetric cell division, to study the mechanism of transformation and other research purposes.

Examples

Example 1: Regulatable activation of the Ras pathway via ligand-mediated association of a targetable Sos protein and membrane docking protein.

MATERIALS AND METHODS

Cell Lines and Transfection Assays. COS cells used in this study were grown in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum and penicillin/streptomycin. Jurkat-TAg cells (16) were maintained in RPMI 1640 supplemented with 10% fetal calf serum, L-glutamine, and penicillin/streptomycin. For transfections, 10^7 Jurkat-TAg cells were electroporated at 960 μFD, 250 V, in 0.4 ml media with the indicated amount of expression plasmids. To assay for SEAP reporter activity, 100 μl of heat inactivated (68 °C, 1hr) samples, transfected and stimulated with the indicated mitogens and FK1012, were added to 100 μl 2M diethanolamine (pH 10.0), 1mM methylumbelliferyl phosphate (MUP), incubated for several hours at 37 °C, and fluorescence units determined with a Titerteck Fluorescan II (ICN) at 355 nm excitation and 460 nm emission.

Protein Analysis. To monitor protein expression, cells were lysed 40 hrs post transfection in RIPA buffer (150 mM NaCl, 20 mM Tris pH 7.5, 0.5% sodium deoxycholate, 0.1% SDS, 1% TritonX100, 1 mM PMSF, 1mM benzamadine, 1μg/ml aprotinin, 1μg/ml antipain, 1μg/ml leupeptin), lysates immunoprecipitated with mAb 12CA5 (BABCO) prebound to protein A-sepharose, analyzed on 10% SDS-PAGE and electrotransferred to nitrocellulose. Blots were probed with 12CA5 and horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Zymed) and proteins visualized by ECL (Amersham). For coimmunoprecipitation assays, transfected Jurkat TAg cells were lysed in HNTG lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 10% glycerol, 1% TritonX100, 1.5 mM MgCl, 1mM EGTA, 1mg/ml BSA, protease inhibitors as above). Lysates were immunoprecipitated with 12CA5 as described above and washed extensively in HNTG buffer (50 mM HEPES, 10% glycerol, 0.1% TritonX100, 150 mM NaCl). Identical immunoprecipitates were analyzed on 10% SDS-PAGE gels and blots probed with 12CA5, or on 12% SDS-PAGE gels and blots probed with anti-Grb-2 mAb (Transduction Laboratories).

Immunofluorescence Microscopy. 50% confluent monolayers of Cos cells were transfected with the indicated plasmid DNAs using LipofectAmine (GibcoBRL) on coverslips. 24 hrs post transfection,

cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% TritonX100 for 5 min, and stained with 12CA5 primary antibody followed by rabbit anti-mouse FITC-conjugated secondary antibody (Pharmingen). Immunofluorescence was visualized using confocal microscopy.

RESULTS

Membrane Localization of Sos in T lymphocytes. Evidence that membrane localization of Sos can activate signaling in T cells was obtained by fusing the v-src myristoylation sequence N-terminal to the coding region of Sos (Figure 1A). A control vector encoding soluble Sos was also constructed in which the myristoylation sequence was eliminated. A C-terminal epitope tag derived from the influenza virus hemagglutinin (HA) was added to the constructs in order to visualize synthesis of the chimeric molecules in cells. Localization of the soluble and myristoylated Sos protein was confirmed in Cos cells by confocal microscopy as specific cell localization/morphology could most easily be observed in this cell line. Expression of the soluble Sos construct was localized exclusively in the cytosol, (Figure 2A, B). Myristoylation of Sos resulted in localization predominantly at the plasma membrane (Figure 2C,D). To assess the role of Sos localization in signaling, these molecules were cotransfected into a human T cell line expressing SV40 large T antigen (Jurkat-TAg cells) for high expression along with a secreted alkaline phosphatase reporter gene under the control of an NF-AT-responsive promoter element (17). NF-AT is a transcription factor that binds regions within the IL-2 enhancer and is essential for transcription of genes such as IL-2, IL-4, GMCSF, and CD40 ligand that coordinate the actions of cells necessary for an immune response. The NF-AT transcription complex responds to signaling through the TCR as well as by pharmacological agents such as phorbol ester and ionomycin, a calcium ionophore, which synergize to activate NF-AT-dependent transcription (18-21). Activation of T cell signal transduction was assayed by induction of NF-AT-dependent transcription of a secreted alkaline phosphatase reporter gene, and accumulation of alkaline phosphatase in the media. Transfection of a constitutively active calcineurin can functionally replace the calcium-dependent events in T cell signaling (17,22,23), while expression of constitutively active Ras (v-Ha-Ras) can bypass the need for phorbol ester stimulation (24). Activities of the soluble and myristoylated Sos constructs were assayed for their ability to provide a signal complimentary to the calcium signal induced by ionomycin stimulation. Either constitutively active Ras or myristoylated Sos synergize with ionomycin to activate NF-AT-dependent transcription in T cells (Figure 1B). In contrast, soluble Sos exhibited only a small activation above the level of vector alone. Similar results were obtained with these constructs in

activating transcription of an AP-1-dependent reporter gene independent of phorbol ester stimulation. The signaling induced by expression of myristoylated Sos was inhibited by cotransfection of dominant negative N17Ras (Figure 1B). These data are consistent with the idea that signaling by membrane-bound Sos is mediated by activation of the Ras pathway. Constitutively active Ras and myristoylated Sos, but not soluble Sos, synergized with constitutively active calcineurin to activate NF-AT in the absense of any additional stimulation (Figure 1C), indicating that Sos functions exclusively on the Ras-dependent pathway of T cell activation. Myristoylated Sos was inactive without either ionomycin stimulation or cotransfection of the calcineurin gene. These data are consistent with recently published observations that membrane localization of full length Sos (26) and of the catalytic domain of Sos (27) in fibroblasts also activates Ras-dependent signaling.

To investigate the specificity of Sos for Ras activation in T cells we compared the activity of Sos to that of the Dbl oncoprotein, thought to be a GEF specific for members of the Rho/Rac subfamily of small GTP binding proteins, including the human CDC42 protein (28-30). In addition, we investigated the activity of the product of the Vav proto-oncogene (31), a hematopoietic-specific protein implicated in a variety of signal transduction pathways, reviewed in (32). Vav was of particular interest since it has been shown to be tyrosine phosphorylated following TCR activation (33), and unconfirmed reports suggests it exhibits a Ras-specific GEF activity following phosphorylation by Lck as well as by diacylglycerol binding (34,35). This is unexpected as the catalytic domain of Vav is homologous to the Rho/Rac family of exchange factors (36), exhibits transformation similar to RhoA and Dbl, and induces phenotypic changes that are distinct from Ras-mediated transformation (37,38). As expected, myristoylated Dbl was unable to activate the Ras-dependent pathway in T cell activation (Figure 1D), indicating that specificity for Ras by a membrane-targeted GEF is essential. Interestingly, expression of the wild-type soluble Vav proto-oncogene (ScVavE) activated NF-AT-dependent transcription in the absense of ionomycin or PMA to levels approximatly 15% of maximal stimulation (Figure 1D), indicating that in contrast to Sos it provides a weak but sufficient signal for NF-AT-directed transcription. Myristoylated Vav (McVavE) did not activate NF-AT-dependent transcription in the absense of ionomycin, and was only slightly elevated following addition of ionomycin. As the McVavE protein appeared to be less stable than ScVavE (Figure 1D, inset), the lowered activity of myristylated Vav may simply be the result of lower protein expression. These results indicate that Vav plays a role in TCR

signaling, manifested by NF-AT-dependent transcription, but that its role appears to be distinct from that of Sos.

The Role of Grb-2 in Ras Activation. To eliminate Grb-2 interaction with myristylated Sos, while maintaining an intact Sos C-terminal domain, the two proline-rich sequences found to be essential for Grb-2 interaction (39) were mutated. The consensus PPR in each domain was changed to AAA in order to abolish interactions with the Grb-2 SH3 domains (Figure 3A). MSosE immunoprecipitates were analyzed for Grb-2 by Western blotting with an anti-Grb-2 antibody. Mutation of the consensus sequence at residues 1147-1156 reduced constitutive Grb-2 binding by approximately 50%, while an additional mutation of the site at residues 1290-1295 eliminated detectable Grb-2 interaction (Figure 3B). This is consistent with the observation that a high affinity Grb-2/Sos interaction requires the coordinate binding of both Grb-2 SH3 domains (39). To identify the role of Grb-2 interactions with Sos, these myristoylated Sos mutants were assayed for their ability to activate Ras-dependent signaling in T cells. The elimination of Grb-2 interaction had no effect on membrane-bound Sos signaling (Figure 3C). This is consistent with a role for Grb-2 in T cells to localize the guanine nucleotide exchange factor Sos to the membrane but not to induce a conformational change. These results demonstrate that Sos can function independent of Grb-2 interaction when constitutively localized to the membrane.

Development of a Conditional Allele of Sos. Constitutive activation of Sos results in prolonged activation of all signals downstream in the Ras activation pathway, potentially initiating feedback pathways and masking immediate effects downstream of Ras. Moreover, imposing membrane-localization on Sos by the addition of a myristoylation sequence may activate its GEF activity by inducing a fortuitous conformational change. To avoid these objections, we devised a conditional allele of Sos in which membrane localization is induced with a rationally designed chemical inducer of dimerization (CID), FK1012, thereby mimicking the role of Grb-2 in membrane-recruitment of Sos (Figure 4A). This allele of Sos was designed by modifying a technique recently developed to induce intracellular oligomerization of cell surface receptors (40,41). Soluble Sos was fused to three domains of FKBP12 (SF3SosE) and coexpressed in Jurkat-TAg cells with membrane-bound myristoylated FKBPs (MF3E). Addition of the CID, FK1012, resulted in the localization of soluble Sos with membrane-bound FKBPs, and the activation of Ras (Figure 4B). Fusion of fewer FKBPs on the myristylated sequence at the membrane or on Sos resulted in less optimal membrane localization, probably due to ineffective interactions of FK1012 with endogenous FKBPs and to homodimerization of MF3Es and SosFKBPs. The

activation induced by soluble Sos recruited to the membrane by FK1012 was inhibited by the coexpression of a dominant negative Ras (Figure 4C). These data provide compelling evidence that the signal induced by the addition of the CID FK1012 is indeed due to the activation of Ras-dependent signaling and that targeting Sos to the plasma membrane per se facilitates the activation of Ras.

Previous work in fibroblasts has indicated that Sos exists prior to stimulation in a previously existing complex with Grb-2. Indeed some Grb-2 can be coprecipitated with Sos in T cells prior to stimulation (Figure 3B), suggesting that localization of Grb-2 to the membrane might also recruit Sos and activate Ras-dependent signals. To assess this possibility in T cells, a modified human Grb-2 molecule containing 3 tandom copies of the FKBP module, SF3GrbE, was generated in the same manner described for the production of SF3SosE (see Figure 1A). Cells were cotransfected with MF3E and SF3GrbE, treated with FK1012 to recruit the modified Grb-2 to the membrane, and stimulated with ionomycin. In contrast to membrane recruitment of Sos, this relocalization of Grb-2 to the membrane did not activate Ras dependent signal transduction in T cells (Fig. 4D). Placing the FKBP module C-terminal to Grb-2 (SGrbF3E) or constiutive localization of Grb-2 to the membrane by addition of an N-terminal myristoylation sequence (MGrbE) likewise did not activate Ras. These results suggest that another level of regulation exists in T cell activation regulating Sos/Grb-2 interaction. Although these data do not rule out that modification of Grb-2 might abrogate its interaction with Sos, this seems unlikely as three separate modifications of Grb-2 did not result in Ras activation.

DISCUSSION

A role for Ras in T cell activation has been clearly established (42), however the mechanism and full consequences of its activation have been the subject of much debate. Our studies indicate that at least one mechanism in T cells may be mediated by membrane recruitment of Sos. Membrane proximity is essential for Ras-dependent events as other components of the Ras pathway such as RasGAP (43) and Raf (44,45) can also be activated by membrane localization. Although Grb-2/Sos membrane-recruitment by the TCR may be mediated by Shc, only low levels of tyrosine phosphorylation on Shc are detected following TCR stimulation (46). In contrast, a 36-38 kD tyrosine phosphoprotein (46,47) that is localized to the particulate fraction of cells has been found associated with Grb-2/Sos following TCR stimulation, and it may be responsible for the

translocation of Grb-2/Sos to the membrane following receptor stimulation. Indeed, recruitment of Sos to the membrane in T cells may be more complex than the localization of a preformed Grb-2/Sos complex to the membrane demonstrated in fibroblasts. Recent work indicates that in T cells the interaction between Grb-2 and Sos is inducible following activation of the antigen receptor, and that this association could be enhanced in vitro by interaction of the Grb-2 SH2 domain with phosphotyrosine-containing peptides from Shc (48). This is consistent with our observation that membrane recruitment of Grb-2 alone, in both the constitutive and inducible systems, did not result in Ras activation.

The possibility that Grb-2 induces a conformational change in Sos and thereby activates its catalytic activity is now unlikely in light of the ability of MSosE to fully activate the Ras pathway in the absense of a detectable interaction between Grb-2 and SOS with mutated SH3-binding regions. This is consistent with data indicating that Grb-2 binding to Sos in vitro has no measurable effect on guanine nucleotide exchange activity (49). Furthermore, biophysical studies have indicated that Grb-2 SH3 domains exhibit no conformational change following Grb-2 SH2 binding to phosphotyrosine peptides (50). In fact, in contrast to the data in mammalian cells, apical membrane localization of Drosophila Sos was shown to be independent of Drk, and an interaction between the Drosophila Sos pleckstrin homology domain and the Sevenless receptor itself was indicated (51). Elimination of the Sos C-terminal domain has been shown to accentuate Sos activity, suggesting that Grb-2 binding to the Sos C-terminus may eliminate a negative regulatory region (26,51). However, the deletion of the Sos C-terminal domain may not activate Sos but rather may eliminate sites of phosphorylation by a MAPK family member which may negatively regulate Sos in a feedback loop (52). Furthermore, deletion of the entire Sos C-terminal domain, normally juxtaposed with the catalytic domain, may induce an errant conformational change resulting in higher catalytic activity or greater accessability of the catalytic domain to Ras.

The role of Vav in TCR activation also remains unclear. An analysis utilizing blastocyst complementation with ES cells containing a mutant vav allele has recently implicated a role for vav in signaling by the TCR (53-55). Although the activation of Vav may require additional signals normally present following TCR receptor stimulation, our data suggest that Vav is not a membrane-recruited GEF for Ras in T cells. The low level of signaling elicited by expression of myristoylated Vav in the presence of ionomycin as well as the low basal activity of soluble Vav in the absence of ionomycin could possibly be generated by recruitment of other signaling

components to the membrane, as Vav contains a variety of motifs important for protein-protein interactions, including SH2, SH3, pleckstrin homology, and leucine-rich domains. Indeed, Vav may also play a role as a Rho/Rac-specific GEF essential for altering components of the cytoskeleton, cell shape, and motility. The precise definition of Vav GEF substrate specificity and the roles of its various domains are needed to define the precise role of Vav in T cell activation.

Our studies are consistent with the hypothesis that Grb-2 functions to link Sos to the cell membrane allowing its constitutive GEF activity to be exerted on membrane-associated Ras. The role of Grb-2 and the rationale for its insertion in the signaling pathway to mediate interaction between the growth factor receptor and Sos may initially be viewed as redundant or baroque. However, this form of inducible linker mediated-dimerization is commonly seen in biology, and we propose that it has two advantages. First, it improves the biologic specificity of the interaction of receptor with Sos since specific contacts must be maintained on either side of Grb-2 to mediate a biologic response. Secondly, linker-mediated dimerization results in more favorable kinetics for signal transduction since the biologic response can be terminated by dissolution of either of the two linkages with Grb-2. If the in vivo dissociation rates of Grb-2 with receptor are similar to those of Grb-2 with Sos, a 2-fold gain in dissociation rate would be realized. At present only solution measurements for individual peptides in vitro are available (56,57), and high asymmetric interactions would reduced the kinetic advantage of this form of mediated dimerization. Other biologic processes that make use of this form of induced dimerization include the heterodimerization of receptors by growth factors such as IL-2 and TGFbeta (58,59), and the facilitated interactions between transcriptional activators and TBP mediated by the TAFs (60,61). To make a conditional allele of Sos we have mimicked this natural form of linker-mediated dimerization by using a rationally designed CID to induce localization of Sos to the membrane.

The use of a conditional allele to inducibly regulate the localization of Sos to the membrane clearly demonstrates that recruitment of Sos alone to the membrane results in Ras activation. Inducible activation also minimizes compensatory or feedback signals that might result from prolonged activation of this pathway, and will allow the temporal dissection of events following both the immediate and prolonged activation of Ras. In addition to the inducible activation of Ras demonstrated here, inducible regulation of members of the Src-like family of tyrosine kinases by membrane localization has also been achieved, illustrating the utility of

CID-mediated protein localization in studying a variety of signal transduction events. This method of ligand induced activation of a GEF illustrates a method that may also be applied to the specific regulation of other Ras-like GTP binding proteins, such as those involved in vesicle transport, secretion, and cytoskeletal rearrangement.

5

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Example 2: Targetable, conditional alleles of Src-family tyorosine kinases

MATERIALS AND METHODS

Plasmid Clones. The expression plasmids used in this study are described in Fig. 5A or below. All of the constructs made by PCR were sequenced. Protein expression was verified by Western blot analysis using the influenza hemagglutinin epitope tag (12CA5) (7). The murine Lck, Fyn and Lyn templates are from m-lck, pmTF and lynAF, respectively (18, 19). Primers were flanked by Xho I (5' primer) or Sal I (3' primer) sites, and the resulting fragments were subcloned into pKS (Stratagene), sequenced, and subcloned into the Sal I site of SF1E (SF1 series) or MF1E (MF1 series) described previously (5). RSV-N17Ras (20) is dominant-negative Harvey Ras mutant.

The reporter plasmids NF-AT-SX, IL-2-SX, AP-1-SX, NFκB-SX and Oct/OAP-SX have been described (5). Briefly, they contain multiple binding sites for the various transcription factors cloned upstream of a minimal interleukin 2 (IL-2) promoter [-70 to +47 (21)] driving secreted alkaline phosphates (SEAP) expression.

Cell Lines and Tissue Culture. Jurkat-TAg cells, J.RT-T3.5 cells (22) and COS (monkey kidney) cells were used for these studies. Jurkat-TAg cells and COS cells contain the simian virus 40 large tumor antigen, which permits replication of plasmids containing the simian virus 40 origin. J.RT-T3.5 is a TCR β-chain-deficient subclone of Jurkat cells (20). All cells were grown and electroporated in RPMI 1640 medium. 10% (vol/vol) fetal calf serum, 10 mM Hepes (pH 7.4) and penicillin/streptomycin.

Electroporations and SEAP Assays. Jurkat-TAg cells were electroporated (Bio-Rad Gene Pulser; 960 μF and 250 V in a 0.4-cm-wide cuvette) with 2-3 μg of the reporter plasmid NF-AT-SX or one of its derivatives (5, 23), 1 μg of the pBJ5 expression vector containing the "docking protein" MF1E or MF3E and 2 μg of pBJ5 containing one of the Src-family kinases or the control construct SF1. Alternatively, 1 μg of MF1Lck was cotransfected with 2 μg of reporter. For COS cells, one-half of the above amounts were used. For J.RT-T3.5 cells, 0.2 μg of expression plasmid RSV-TAg (24) was cotransfected to increase expression. After 24 hr, aliquots of cells were stimulated with dilutions of FK1012A or mitogen (1 μm ionomycin plus phorbol 12-myristate 13-acetate and 25 ng/ml) plus 1 μM nonreactive, monomeric control ligand FK506-M (5). After 20 hr, supernatants were assayed for SEAP activity as reported (5) and the data are presented relative to mitogen

stimulation. Each data point was performed in duplicate, and the data are the average of two experiments. All reagents were dissolved in ethanol and the maximum concentration of solvent in culture never exceeded 0.1%.

Kinetics of Activation. Anti-TCR monoclonal antibody (mAb) UCHT1 (Sigma)-coated microtiter wells were incubated for 1 hr at 37°C with 10 μg of mAb per ml of phosphate-buffered saline (PBS) and blocked for 1 hr 37°C in PBS containing 1% fetal calf serum. Cells were divided into these wells or untreated wells containing mitogen or FK1012. Aliquots of cells were removed from the microtiter wells at each time point and frozen until the last time point. The data are presented as the average of two experiments performed in duplicate.

RESULTS

To be able to reversibly recruit the Src kinases to the membrane, we removed the myristoylation-targeting peptides from the N termini of Fyn, Lck and Lyn and replaced them with FKBP12 (Fig 5A) (8, 25). We also designed a membrane-bound docking protein, MFnE, composed of the N-terminal myristoylation targeting peptide from v-src followed by n = 1-3 copies of FKBP12 (5). To focus on downstream signaling events, we reasoned that we needed to eliminate the influence of upstream regulatory events [e.g., Csk phosphorylation and CD45 dephosphorylation (Fig. 5B) in kinase activation. Therefore, chimeric Lck (SF1Lck), Fyn (SF1Fyn) and Lyn (SF1Lyn) were deregulated by eliminating their regulatory, C-terminal tyrosine residues (Fig 5A). A priori, the administration of the CID FK1012 (5, 26) should lead to the formation of hetero- and homodimers (Fig 5C).

To demonstrate the feasibility of this approach, Jurkat-TAg cells (24) were cotransfected with one of several FKBP-12-containing constructs and a reporter plasmid (NF-AT-SX) in which the SEAP gene is under the control of the NF-AT transcription factor (23, 27). NF-AT-dependent transcription of this reporter is elicited by the antigen receptor through a bifurcating signaling pathway (28-30) that requires both calcineurin and Ras and that is inhibited by cyclosporin A and FK506 (31). Thus, in several respects, it faithfully mimics the activation requirements of T lymphocytes. When the cytosolic FKBP12-Fyn chimera (SF1Fyn) or the docking protein (MF1E) is transfected into Jurkat-TAg cells, NF-AT activity is undetectable at all concentrations of FK1012 (Fig. 6A). However, if the docking protein and the FKBP12-Fyn chimera are cotransfected into Jurkat-TAg cells, FK1012 activates signaling at concentrations as low as 1 nM.

Membrane recruitment of "wild-type" Fyn, SF1Fynwt, is insufficient for signaling (Fig. 6A). As expected, FK1012-mediated membrane recruitment and crosslinking of SF3Fynwt, which contains three FKBP12s, signaled effectively. Also, membrane-tethered, deregulated Fyn (MF1Fyn) activated signaling in an FK1012-independent fashion (≈40% relative to ionomycin and phorbol 12-myristate 13-acetate activation (refs. 7 and 9).

To investigate potential specialization among several Src family members, we compared the ability of Fyn, Lck and Lyn to activate signaling. Lck activates slightly, but reproducibly, better in this system at lower FK1012 concentrations than Fyn or Lyn, whereas there is no significant difference in the efficiency by which membrane recruitment of deregulated Fyn or Lyn activates signaling (Fig. 6B). Although Lyn is predominately B-cell specific (32), it can apparently signal in T cells when artificially targeted to the plasma membrane. These results suggest that the kinase domains of Src family members may not contribute significantly to their specific roles but that specific mechanisms of activating these kinase are important for their biologic specificity.

We investigated the role of the Src homology (SH) 2 and 3 domains in signaling induced by FK1012 by constructing a series of Fyn and Lck mutants lacking one or both SH3 and SH2 domains. MF3E was cotransfected into Jurkat cells alone with one of the various Fyn or Lck constructs or kinase-deficient mutants (Figs. 5A and 6C and D). These results indicate that the kinase domains are not only necessary but also sufficient for signaling, whereas neither the SH3 nor the SH2 domain is required for the induction of signaling and, in fact, may be inhibitory. The dispensability of these N-terminal domains rules out the formal possibility that membrane recruitment of SH3- or SH2-associated signaling molecules is responsible for signaling.

As previously shown, crosslinking the intact TCR or the ζ chain of the TCR complex activates a subset of transcription factors necessary for the transcriptional induction of the IL-2 gene, including NF-AT, Oct/OAP and AP-1 whereas activation of NF-κB factors requires costimulatory signals (5, 33). To assay whether inducible Fyn or Lck can activate a similar panel of factors, we cotransfected various reporter plasmids into Jurkat-TAg cells along with MF3E and SF1Fyn or SF1Lck (Fig. 7A and B respectively). Upon addition of FK1012, only the NF-AT-, Oct/OAP- and AP-1-responsive reporters were activated, whereas the NF-κB-responsive and the IL-2 enhancer-containing reporter were completely inactive. Additionally, NF-AT-dependent transcription induced by Fyn or Lck is blocked by FK506, a potent inhibitor of the Ca2+-regulated phosphatase calcineurin (23, 34, 35) and requires functional Ras activity, because

a dominant-negative Ras, RSV-N17 Ras, completely blocks induction (Fig 7C) (20). Finally, we examined the kinetics of activation by Fyn and Lck and compared them to that of direct TCR stimulation or mitogen stimulation. Although the total levels of reporter enzyme secretion are severalfold higher by TCR-stimulated cells than by Fyn or Lck-stimulated cells, in either case, reporter activity is first measurable at 3 hr, demonstrating the FK1012-activated signaling is comparable to that of the receptor (Fig 7D). Therefore, by multiple criteria the induction of Fyn or Lck by FK1012 seems to reproduce TCR-mediated signaling faithfully.

To investigate the dependence of Fyn or Lck signaling on the TCR complex, SF1Fyn or SF1Lck plus MF3E were transiently transfected into the TCR- subclone of Jurkat cells, J.RT-T3.5 (22). Since these cells lack the TCR β chain, they do not assemble a TCR complex at the plasma membrane, resulting in the enhanced degradation or retention in the endoplasmic reticulum of the unassembled TCR complex subunits (36). Surprisingly, membrane recruitment of Fyn (Fig. 8A) or Lck initiated signaling in the absence of a functional TCR. To investigate this, we transfected SF1Fyn or kinase-deficient SF1FynΔK plus MF3E into nonlymphocyte COS cells (Fig. 8B). Again, membrane recruitment of Fyn was sufficient for signaling, whereas the kinase-deficient SF1FynΔK was defective in its ability to signal. These results indicate that signaling by Src family kinases may be able to bypass the antigen receptor complex.

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Claims

1. A genetically engineered cell containing and capable of expressing recombinant DNA sequences encoding a first multimerizable chimeric protein and a second multimerizable chimeric protein,

each of which chimeric proteins contains a domain, which may be the same or different, which is capable of binding to a predetermined multivalent ligand to form a multimer comprising the chimeric protein molecules and the multivalent ligand to which they are capable of binding,

wherein in the presence of the ligand

(a) the genetically engineered cell regulatably expresses at least one DNA sequence comprising a transforming gene,

(b) the multimer binds to the cell membrane, or

(c) the multimer links a cellular destruction domain to a tumor suppressor or a viral protein.

2. A genetically engineered cell of claim 1 which is of mammalian origin.

3. A genetically engineered cell of claim 2 which is of human origin.

4. A genetically engineered cell of claim 2 which is not terminally differentiated.

5. A genetically engineered cell of claim 1 which contains recombinant DNA sequences encoding two or more different chimeric proteins.

6. A genetically engineered cell of claim 1 which contains one or more genes under the transcriptional control of at least one DNA element responsive to the presence of the ligand, in which cell said genes are regulatably expressed in the presence of the ligand.

7. A genetically engineered cell of claim 6 in which at least one of said genes is a transforming gene, a gene for a membrane-targeted transforming gene product or a membrane-targetable transforming gene product.

8. A genetically engineered cell of claim 1 which contains and is capable of expressing a first recombinant DNA sequence encoding a chimeric protein capable of binding to a target protein and to a multimerizing ligand and a second recombinant DNA sequence encoding a chimeric protein containing a degradation targeting domain and a domain capable of binding to the multimerizing ligand

9. A genetically engineered cell of claim 1 which contains and is capable of expressing recombinant DNA sequences encoding two chimeric proteins, each of which chimeric proteins contains a domain capable of binding to a multimerizing ligand, the first chimeric protein also containing a domain capable of binding to a tumor suppressor or viral protein and the second chimeric protein also containing a degradation targeting domain.

10. A recombinant DNA sequence encoding a chimeric protein comprising a receptor domain capable of binding to a predetermined ligand and:

(a) a transforming gene product,

(b) a domain capable of binding to a tumor suppressor or viral protein,

(c) a membrane targeting domain, or

(d) a targeting domain for cellular degradation.

11. A chimeric protein encoded by a recombinant DNA sequencce of claim 10.

12. A recombinant DNA sequence comprising a transforming gene or transforming portion thereof linked to a membrane targeting signal.

13. A chimeric protein encoded by a recombinant DNA sequencce of claim 12.

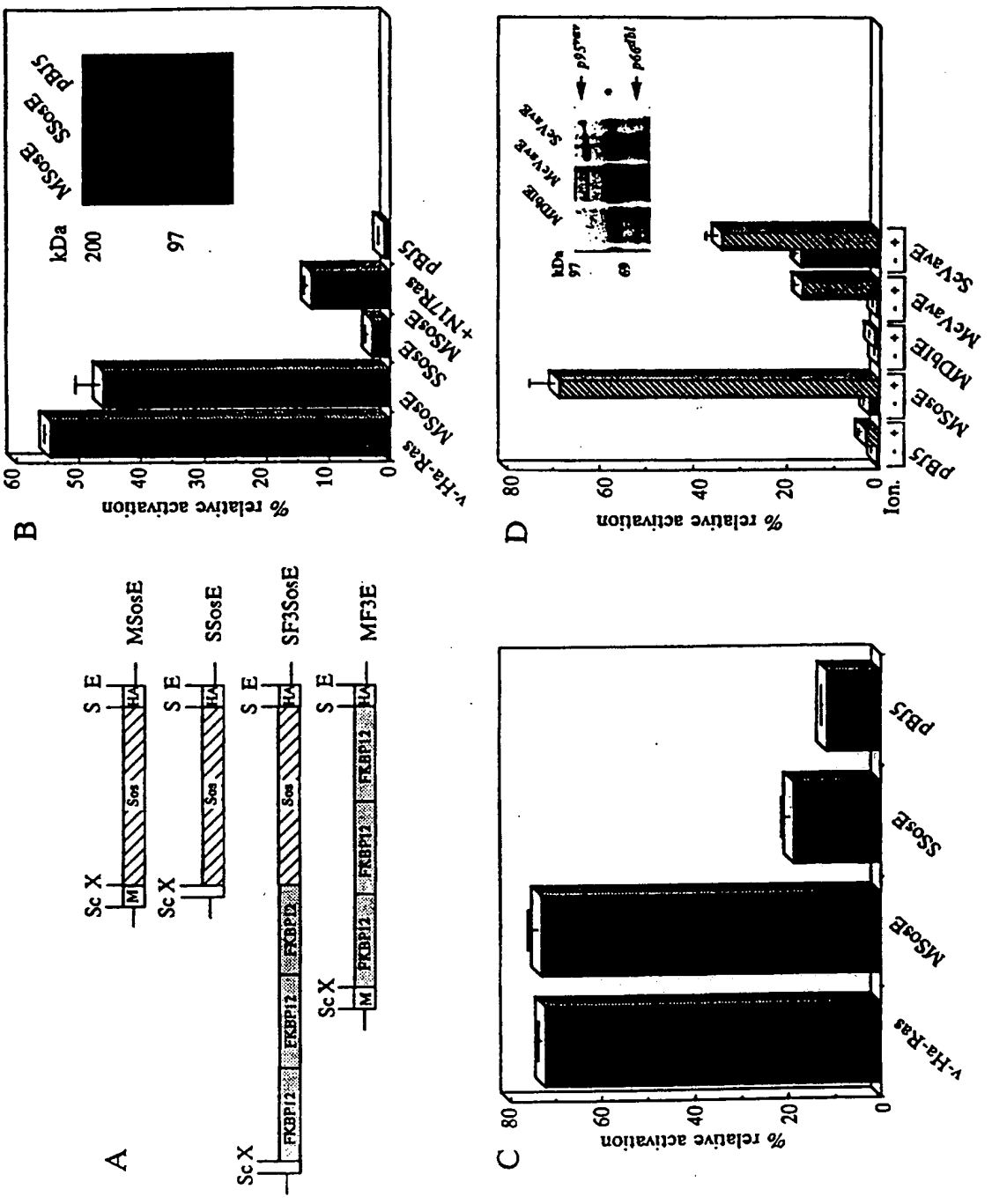
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Figure 1

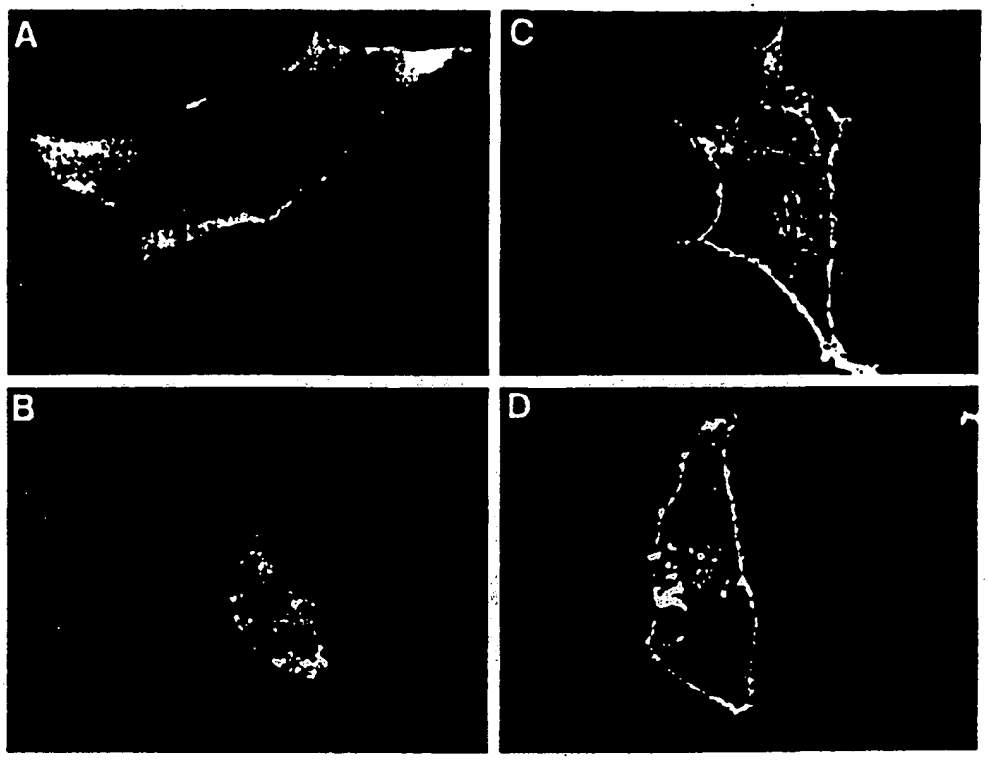
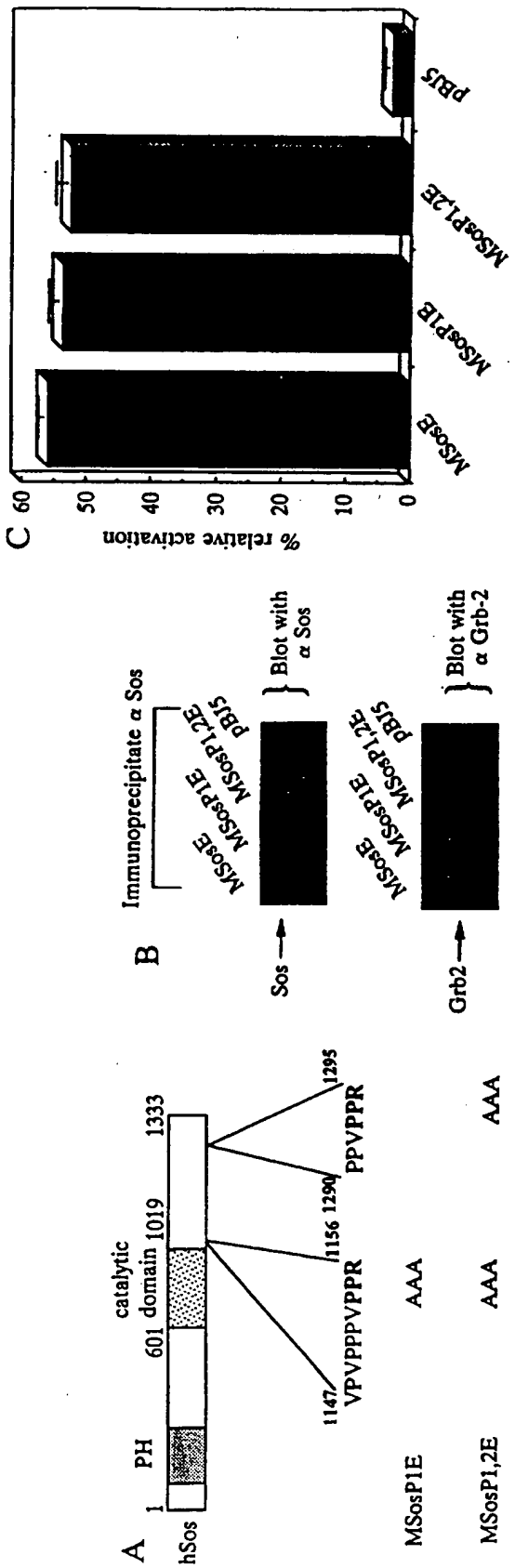


Figure 2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MSosP1,2E
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Blot with α Grb-2
Grb2
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PH
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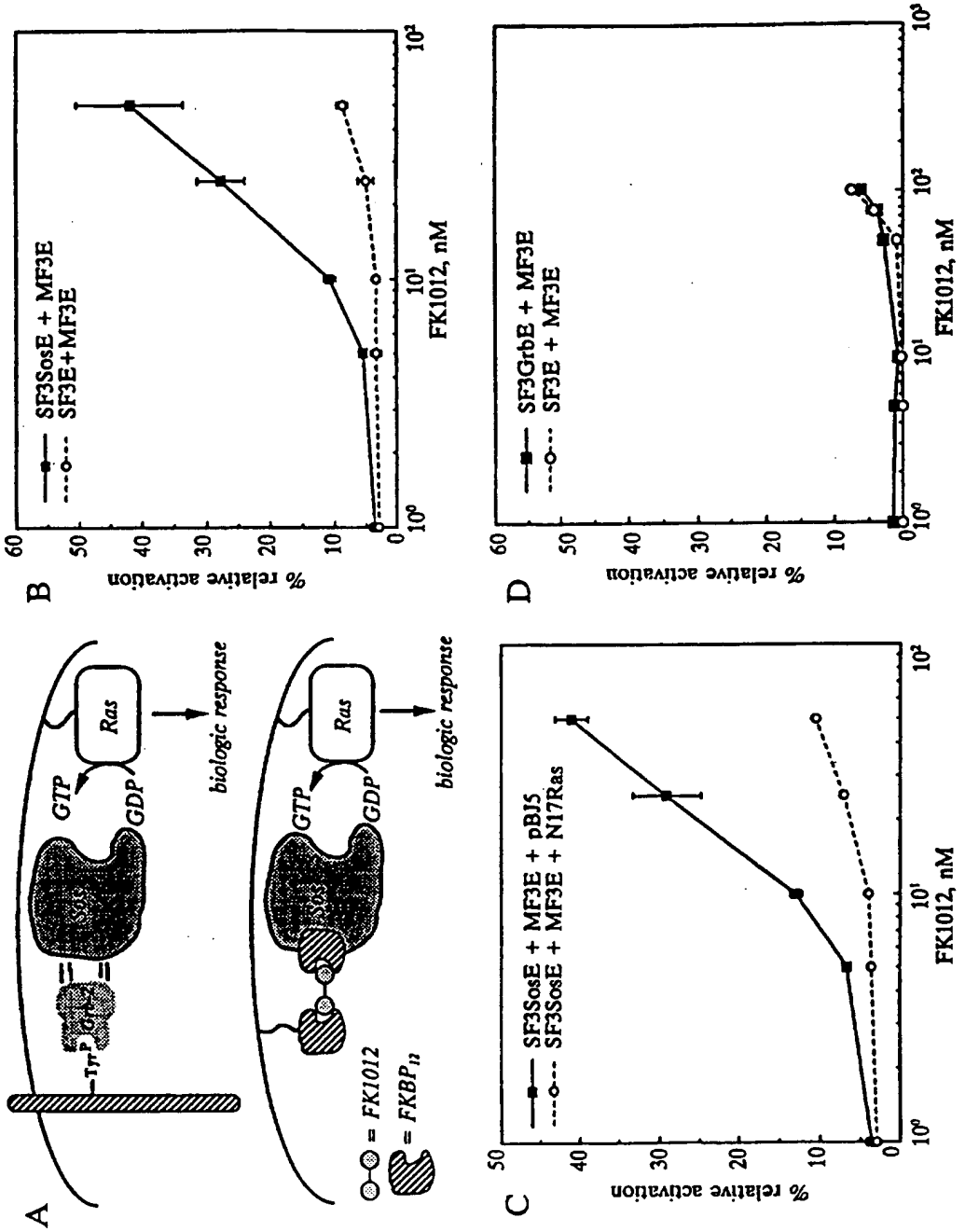
Figure 3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C
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Figure 4

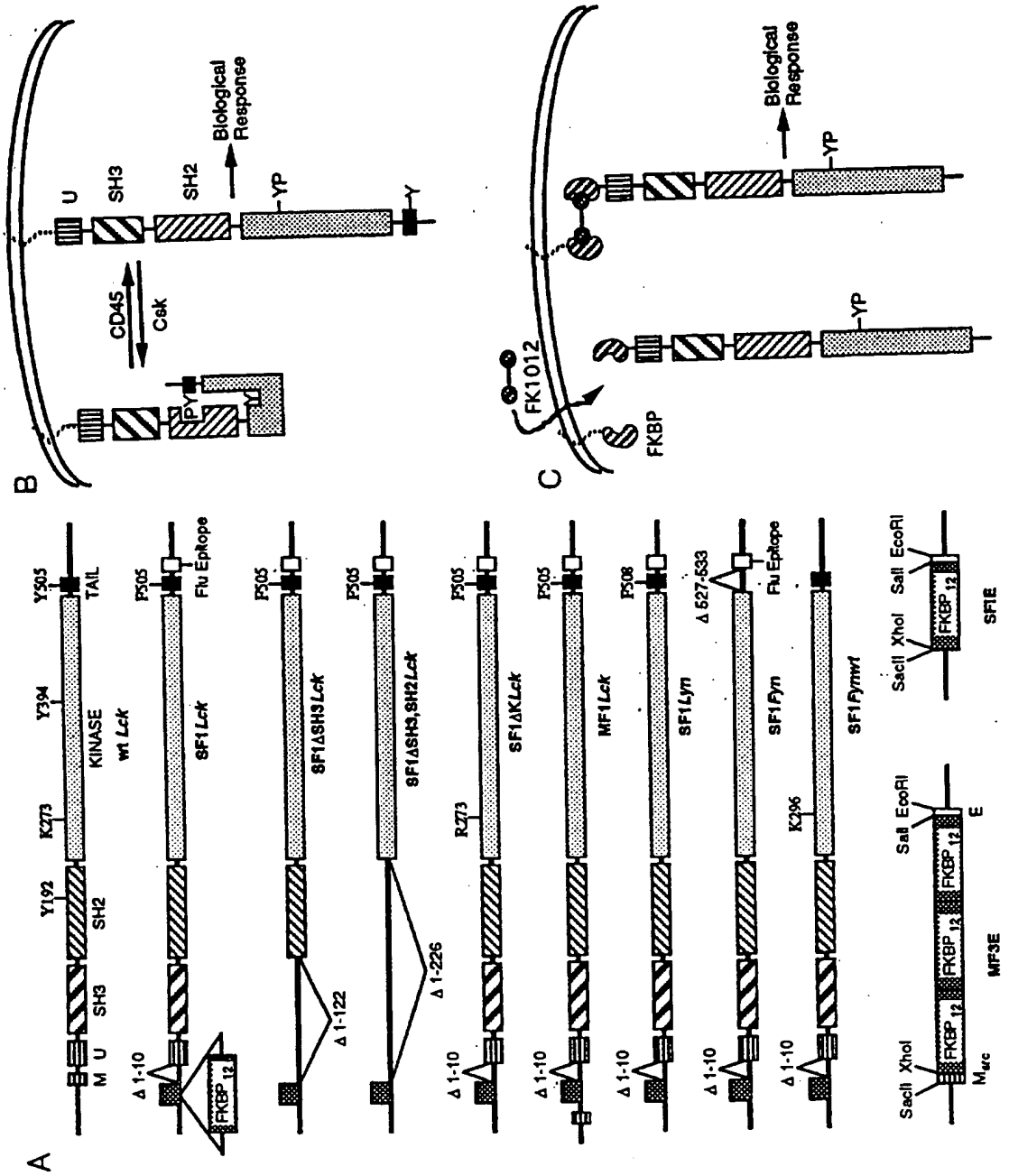
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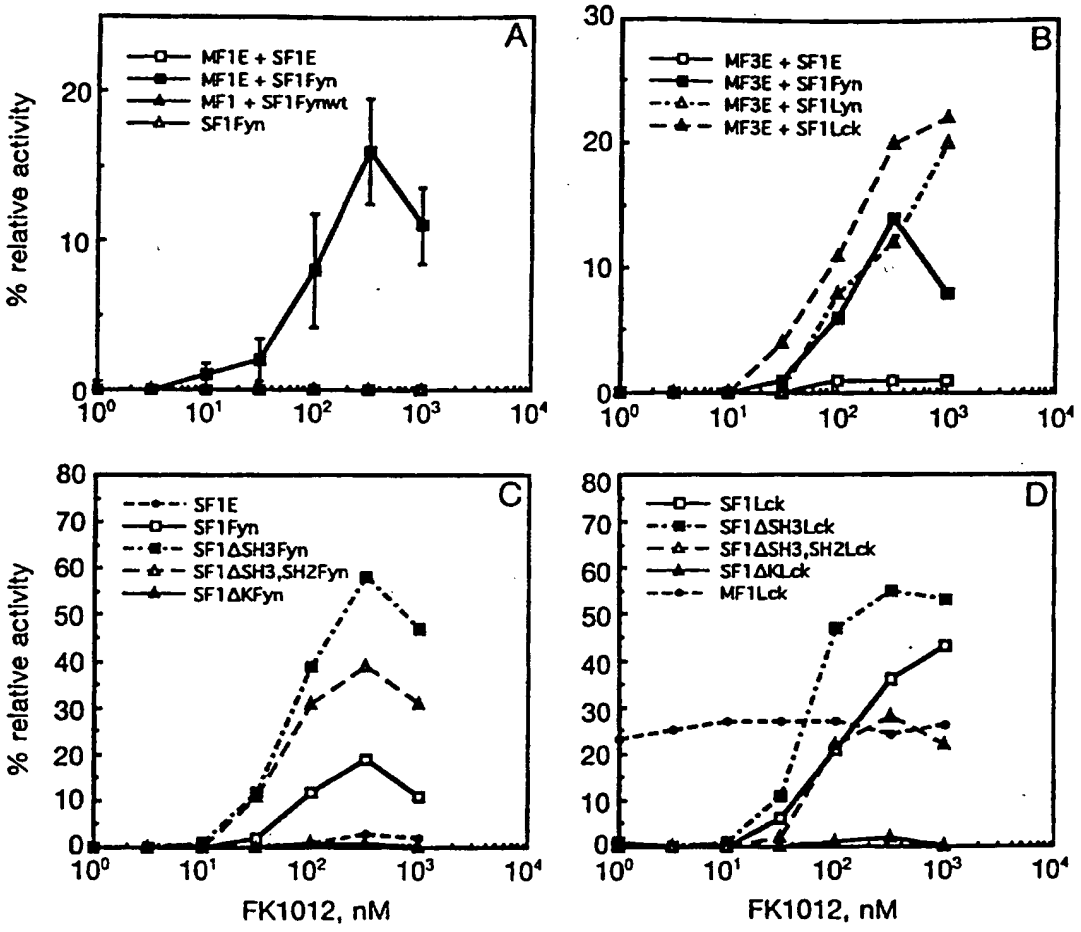
Figure 5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MF3E + SF1Lck
C
SF1E
SF1Fyn
SF1ΔSH3Fyn
SF1ΔSH3,SH2Fyn
SF1ΔKFyn
% relative activity
FK1012, nM
D
SF1Lck
SF1ΔSH3Lck
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MF1Lck
FK1012, nM

Figure 6

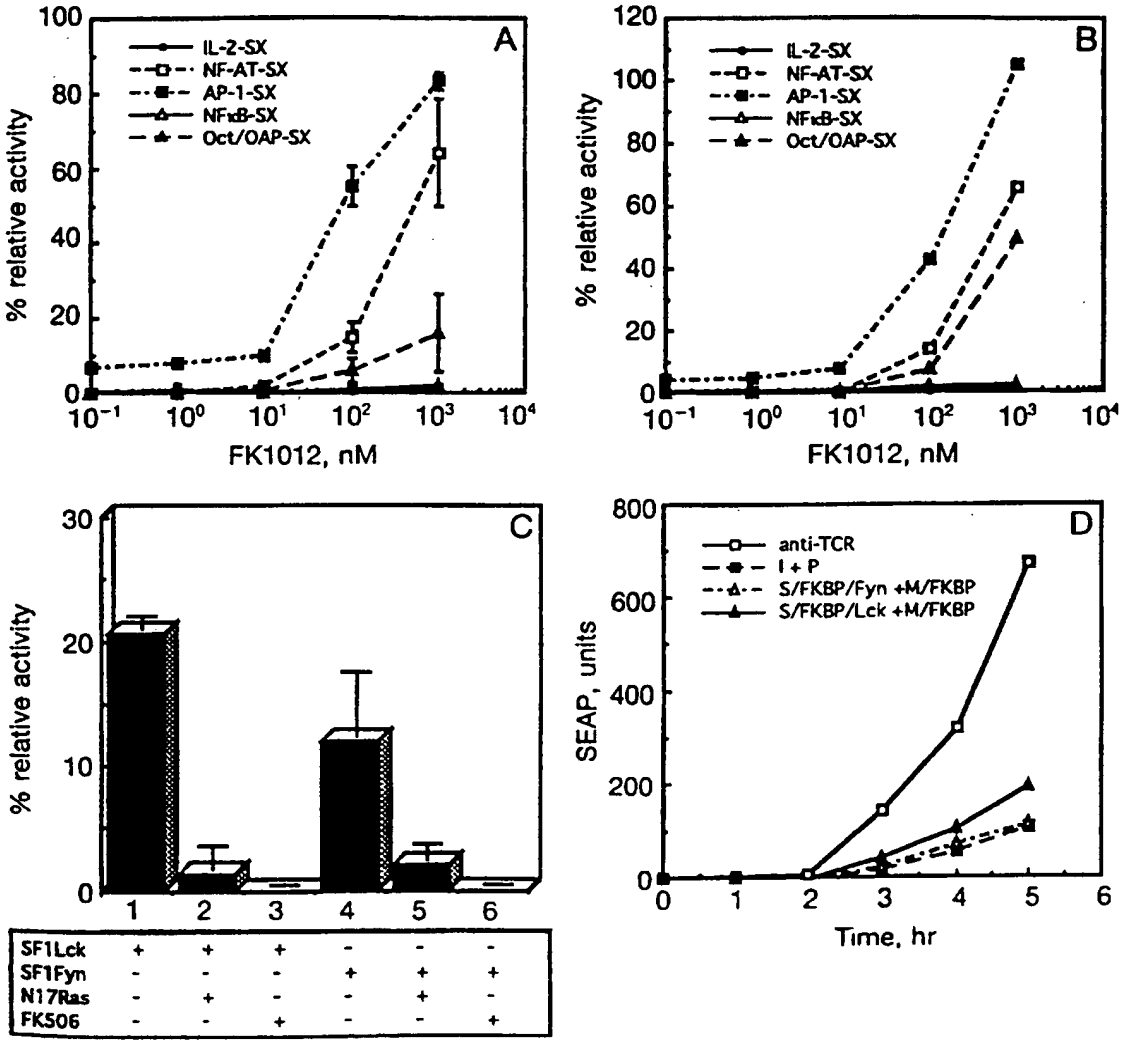
A
IL-2-SX
NF-AT-SX
AP-1-SX
NFκB-SX
Oct/OAP-SX
% relative activity
FK1012, nM
B
C
SF1Lck
SF1Fyn
N17Ras
FK506
D
anti-TCR
I + P
S/FKBP/Fyn +M/FKBP
S/FKBP/Lck +M/FKBP
SEAP, units
Time, hr

Figure 7

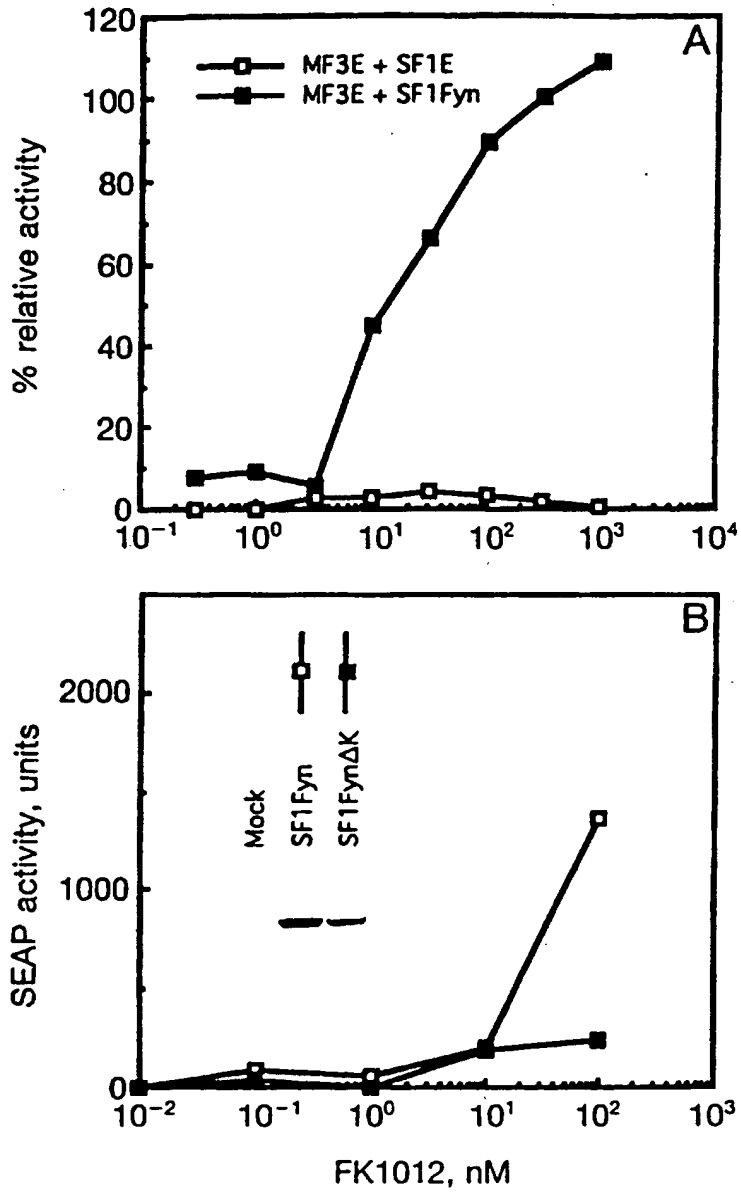
A
MF3E + SF1E
MF3E + SF1Fyn
% relative activity
120
100
80
60
40
20
0
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
10^{4}
B
SEAP activity, units
2000
1000
0
Mock
SF1Fyn
SF1FynΔK
10^{-2}
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
FK1012, nM

Figure 8

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13776

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 5/10, 15/12; C07K 14/47, 14/705
US CL :435/240.2; 536/23.4; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/240.2; 536/23.4; 530/350

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X --- Y	Science, Volume 262, issued 12 November 1993, Spencer et al., "Controlling Signal Transduction with Synthetic Ligands", pages 1019-1024, see entire document.	1-5, 10, 11 --- 6-9, 12, 13
Y	Biological Abstracts, Volume 88, Number 10, issued 15 November 1989, Klemenz et al., "The v-mos and c-Ha-ras oncoproteins exert similar effects on the pattern of protein synthesis", page AB-680, column 1, abstract no. 109986, Oncogene, 4(6), pages 799-804, see entire abstract.	1-13

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:	
"A" document defining the general state of the art which is not considered to be part of particular relevance	
"E" earlier document published on or after the international filing date	
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	
"O" document referring to an oral disclosure, use, exhibition or other means	
"P" document published prior to the international filing date but later than the priority date claimed	
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	
"&" document member of the same patent family	

Date of the actual completion of the international search 18 JANUARY 1996	Date of mailing of the international search report 12 FEB 1996
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer JAMES KETTER Telephone No. (703) 308-0196

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US95/13776

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Biological Abstracts, Volume 97, Number 5, issued 15 March 1994, Pierce et al., "Inhibition of mammary duct development but not alveolar outgrowth during pregnancy in transgenic mice expressing active TGF-β1", page AB-469, column 1, abstract no. 56404, Genes & Development, 7(12A), pages 2308-2317, see entire abstract.	1-13
Y	Biological Abstracts, Volume 85, Number 5, issued 01 March 1988, Trimble et al., "Morphological transformation and tumorigenicity in C3H/10T1/2 cells transformed with an inducible c-Ha-ras oncogene", page AB-445, column 1, abstract no. 47285, Biosci. Rep., 7(7), pages 579-586, see entire abstract.	1-13